WILEY AWARD ADDRESS

Analytical Supercritical Fluid Techniques and Methodology: Conceptualization and Reduction to Practice

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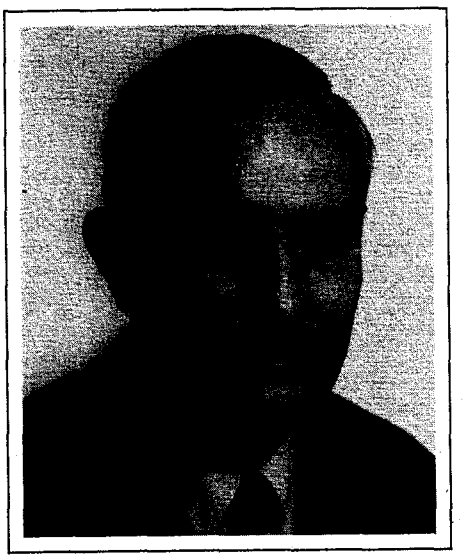
The Wiley Award address, which I have been selected to deliver, culminates a 6-year period of activity within AOAC, in which analytical supercritical fluid techniques have been discussed and advocated. Starting with the 105th annual AOAC Meeting held in Phoenix, Arizona, in August of 1991, there have been 4 additional AOAC-sponsored symposia that conveyed the potential usefulness of this technology for isolating and detecting an assortment of analytes such as drugs, pesticides, pollutants, and nutrients in a myriad of sample matrixes. It is particularly gratifying to me as a researcher in this field to receive the Harvey Wiley Award, because its namesake was an early faculty member in the 1800's at Butler University, my alma mater in Indianapolis, Indiana.

In this address, I shall attempt to answer several vexing questions. Why did interest in the particular technique of supercritical fluid extraction (SFE) occur at this point in time, and what advantages and savings are realized by utilizing it for chemical analysis? In addition, why has our research group at the U.S. Department of Agriculture (USDA) been particularly successful in exploiting the technology for analytical purposes, which we believe has influenced the design of instrumentation and the way in which SFE is practiced? Aside from these seminal issues, I shall also note how we and others have integrated sample cleanup and reaction chemistry along with SFE to provide sophisticated techniques that save both time and money in the analytical laboratory. And finally, because I am affiliated with a federal laboratory whose mission is research and development in the field of agriculture and food science, I have chosen to discuss in detail the saga of analytical SFE as it pertains to fat analysis in foodstuffs, a topic of current interest to both scientists and consumers alike. Hopefully this approach will encourage other analysts to explore some of the benefits of the "supercritical state" in analytical chemistry.

Presented at the 111th AOAC INTERNATIONAL Annual Meeting, September 7–11, 1997, in San Diego, CA.



The Case Against Organic Solvents

As noted by Hawthorne (1), the Soxhlet extraction technique and its many variants have been in use since 1906. This technique and the classic liquid–liquid separatory funnel partition methods have been the dominant techniques used by analysts to isolate target analytes from sample matrixes for over 90 years. Accompanying these techniques has been the use of a plethora of organic solvents as extractants or partitioning phases. However, beginning in 1990, regulatory legislation, such as the U.S. Environmental Protection Agency's (EPA) Pollution Prevention Act; the Superfund Amendments and Reauthorization Act (SARA); the Resource, Conservation and Recovery Act (RCRA); and the Montreal protocols have critically focussed on a reduction in use of specific organic solvents that are harmful to the environment (2).

Aside from these formal mandates, there is good reason to consider the elimination or minimization of organic solvent use in the chemical laboratory. Practical everyday experience suggests that if alternatives could be found to using organic solvents for extraction

or partition in chemical analysis, the following could be minimized: storage and disposal costs associated with used solvents; purchase and storage of high purity organic solvents; exposure of laboratory personnel to harmful solvents; problems associated with collection/storage of waste solvents; tracking of waste solvents (i.e, cradle-to-grave responsibility); disposal of used solvent containers; and jettisoning solvents into the environment.

SFE is but one of several alternative methods that addresses some of the above concerns. Other techniques or practices that can eliminate or substantially reduce our dependence on organic solvents in the laboratory environment are a reduction in sample size, use of a form of "modified" water (3) which we shall discuss later, solvent-limiting techniques such as solid-phase microfibre extraction (4), solid-phase extraction (5), membranes (6), or immunoassay (7). It would also be fair to say, that overall today, there is a trend towards the miniaturization of previously developed methods that reduces reagent use during chemical analysis.

Aside from the ecological benefits of using supercritical fluids, particularly supercritical carbon dioxide ($SC-CO_2$) (8), there are other advantages of SFE. Mass transport properties, such as fluid and analyte diffusion coefficients in supercritical fluids, are greater in supercritical fluid media than in conventional liquid solvents (9), resulting in faster extraction fluxes and a substantial reduction in extraction times. Replacement of a high quality organic solvent by $SC-CO_2$ can also result in cost savings, particularly if one elects to purify an industrial grade of CO_2 (10). Supercritical carbon dioxide also provides a safe, nonflammable medium, devoid of the presence of oxygen, in which to conduct extractions of thermally labile and oxygen-sensitive analytes. Overall, the complexity of an analytical method may also be reduced.

An excellent example of the last mentioned benefit is illustrated in Figure 1, where use of SFE coupled with enzyme immunoassay (EIA) results in a more rapid, quantitative assay for determination of carbamate pesticides in meats. As shown in Figure 1, the conventional method used by the USDA Food Safety and Inspection Service (11) entails use of carcinogenic methylene chloride in the initial extraction step, followed by a series of filtration and concentration steps, 2 chromatographic-based sample cleanup steps, membrane filtration, followed by the final analytical determination of the pesticide by high performance liquid chromatography (HPLC) using fluorescence detection after sample hydrolysis and derivatization is affected. A method developed by Nam and King (12) provides a much simpler alternative as shown in Figure 1, where $SC-CO_2$ accompanied by a small amount of organic modifier is collected in water–methanol, filtered and then diluted with an aqueous buffer solution, and membrane-filtered before applying a commercial

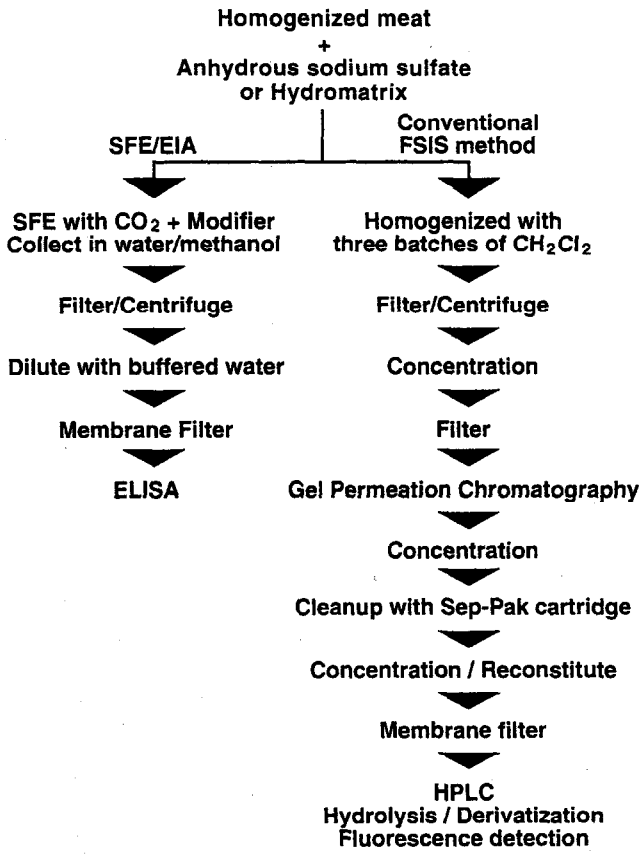


Figure 1. Analytical methodologies for determination of carbamates in meat samples.

ELISA test. This method saves considerable time, uses primarily water and CO_2 as solvents, and can be used in a food production facility.

The Development of Instrumentation and Technique in Analytical SFE

There are different approaches to developing a new analytical technique and advances made by one research group do not take place in a vacuum devoid of influences from others working along the same lines. However, our approach has been somewhat unique in that we had an established program in "process" SFE that had existed in our research group for sometime and this was to play a vital role in conceptualizing our approach to "analytical" SFE. As shown in Figure 2, there exists a synergism in our efforts between analytical method development and process development which has served us well. More analytical chemists would do well to consult the engineering literature (and vice versa) for theoretical principles to guide method development, for pertinent physicochemical data, and as an aid in selecting the appropriate scale for conducting analytical SFE. This process can be reversed to provide equipment and methods that are equally applicable to bench scale process development (13). The reader is encouraged to consult several tomes on chemical engineering with supercritical fluids (14–16) as an

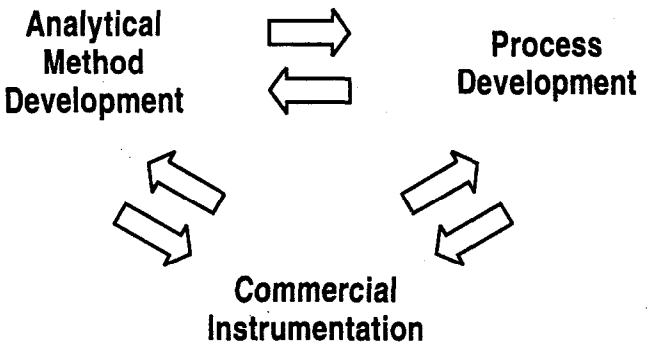


Figure 2. Triangular research synergism in the supercritical fluid technology program at NCAUR.

aid in her/his method development. Using this approach, it is worth commenting on our role in developing commercial instrumentation for SFE.

Today's analytical SFE instrumentation represents a hybrid of different influences, but there is no doubt that it is primarily designed for off-line SFE (17). Table 1 lists the various desired features sought by analysts in commercial SFE instrumentation. As we advocated several years ago (18), most instrumentation should have an upper pressure and temperature range of 10 000 psi and 150°C, respectively. A flow rate minimum of 2–4 L/min (expanded gas flow) is also highly desired, particularly for conducting exhaustive extractions (i.e., defatting) in minimal time. An acceptable sample size range is more difficult to define, because each analytical problem has its own requirements, but most commercial extractors have at least a 10 cc cell capacity which will accommodate 5–10 g of material.

Levy (19) has nicely summarized collection options; namely open vessel, sorbent cartridge, and collection in a liquid; all with or without cryogenic cooling. However, to date, no one company has produced an instrument that embraces all of these extract collection options or that easily allows the substitution of one for the other. We have found that with some ingenuity, the analyst can make subtle modifications to existing commercial instrumentation to affect most of the above collection options (20).

Instrument manufacturers for the most part have attempted to produce small "footprint" equipment that is compatible with limited laboratory bench space. This goal, however, has had an adverse effect in limiting sample sizes that can be processed via analytical SFE. Instrument portability becomes an issue if on-site or in plant analysis is desired, but so far the demand for this feature has been limited among commercial users, although one company has produced a portable module for field use. Automation in analytical SFE has been addressed in two contrasting ways; one embodying sequential extraction of samples in series, while other units have utilized parallel sample processing. Sequential extractors exist that can process up to 44 samples consecutively, while parallel extractors depending upon how they are configured, can usually extract up to 6 samples at one time.

Table 1. Analytical SFE—desired features

- Pressure, temperature, and flow rate ranges
- Sample size range
- Variety of collection options
- Size and portability of instrument
- Automation
- Cosolvent capability
- Ability to interface with other instruments
- Delivery system for carbon dioxide

A comment should be made on the parallel mode of extraction because our laboratory has been involved in developing instrumentation for this mode of extraction. Several years ago we designed a multiple sample extractor that could extract 6 samples in parallel simultaneously (21). This was partly to emulate commercially available Soxhlet-based extractors offered on the market place at that time. This prototype unit underwent several iterations with the assistance of Marvin Hopper of the FDA laboratory in Lenexa, Kansas, where such a unit is currently used along with commercial instrumentation as part of a standard operating procedure (SOP) for the analysis of pesticide residues in a variety of food samples (22). These home-built units had the additional feature of being able to accommodate relatively large samples, i.e., up to 100 g sample on an individual basis at one time. Several commercial units based on this principle were also subsequently offered (23), but were compatible with much smaller sample sizes. These instruments in one case consisted of a unit that could accommodate 8 samples in parallel, while other units relied on "piggybacking" individual extractors with 2–3 sample capacity, to expand a laboratory's extraction capability.

Finally, returning to Table 1, cosolvent capability has become a desired feature because some analyses require small quantities of cosolvents to address analyte solubility limitations in the supercritical fluid, or as an aid in accelerating the extraction from specific sample matrixes (24). It is worth noting that with the capability of adding the cosolvent to a supercritical fluid, the analyst can explore the potential of enhanced fluidity extractions pioneered by Olesik and coworkers (25), or if needed, one of the forms of high temperature (and pressure) liquid extraction (26). Delivery systems for CO_2 largely remain cylinder-based, although this author has advocated a laboratory-wide distribution system for a multitude of supercritical fluids applications in the laboratory (13).

Are There Alternative Fluids to Carbon Dioxide?

In both analytical and process SFE, SC–CO_2 has reigned supreme. However, there are signs in both application areas of supercritical fluid technology that

$SC-CO_2$ is being challenged. In analytical SFE, it has been hard to find a fluid that has as convenient a critical temperature and pressure as CO_2, coupled with its relative nontoxic nature, high non-ideality as a fluid, and its relative benign nature toward most analytes. There are a limited number of cases where CO_2's isoelectronic cousin, N_2O, has found application (27), but its role as a potent oxidizer has been noted with disastrous consequences by Raney (28). A host of new fluorocarbon fluids seems to hold promise, particularly in the area of environmental analysis (29).

Fluoroform (trifluoromethane or HCF_3) continues to hold a fascination for analytical chemists, since Stahl first cited its use (30), due to its similar critical constants to CO_2, and associated dipole moment and hydrogen-bonding propensity. The author, based on Stahl's initial studies, first applied HCF_3 for "inverse SFE" (31), noting that fluoroform could be made selective for target analyte in the presence of potential lipid coextractives. Further studies by Ashraf-Khorassani et al. (32, 33) have shown the above principle to be of value when extracting such moieties as drugs and pesticides in lipid-containing matrixes.

Recently, use of water in its subcritical state has been demonstrated to be effective for the extraction of nonpolar analytes, such as polycyclic aromatic hydrocarbons (34). In this case, the fluid is being used under its critical temperature and pressure, as indicated in Figure 3, in the liquid region under pressure. Clearly this is outside the region normally designated as a supercritical fluid (the solid-lined region in the upper right hand corner of Figure 3). This is similar to the technique frequently noted as accelerated solvent extraction (ASE). It is worth noting that the definition of the supercritical fluid region is somewhat arbitrary as Chester (35) has noted. The true phase diagram in Figure 3 has no such arbitrary boundaries to define the "supercritical" region; therefore, one can readily go from one physical state to the other should it prove advantageous.

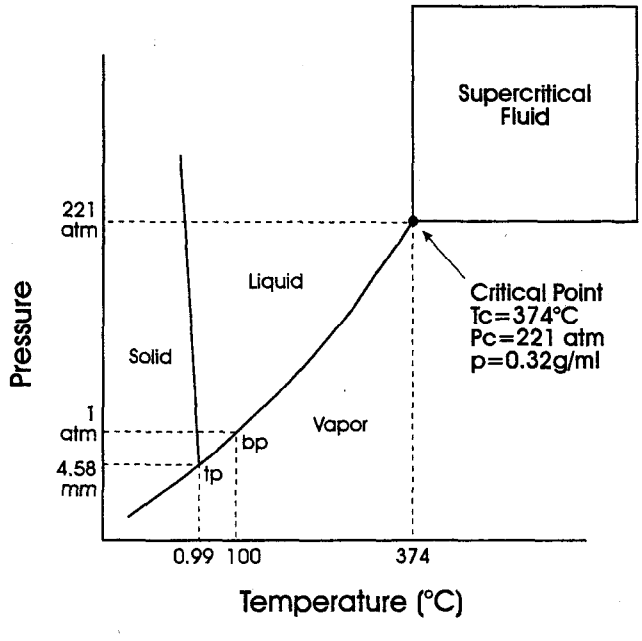


Figure 3. Pressure–temperature phase diagram for water.

Such is the case for subcritical water. Water at temperatures higher than its normal boiling point at atmospheric pressure and under pressure exhibits solvation properties that will permit it to function as an extraction agent for both nonpolar as well as polar solutes. For example, subcritical H_2O dielectric constant can range from 78.5 C^2/NM^2 to approximately 5 C^2/NM^2 in its supercritical fluid state. This latter value approximates the dielectric constants associated with such conventional liquid solvents as diethyl ether and ethyl acetate, which are often used for the extraction of nonpolar analytes. Hence, by adjusting the extraction temperature of water under pressure, a range of solubility behaviors can be mimicked. This property has been recently put to good use to selectively extract different hydrocarbon species with subcritical water from soil matrixes by Yang et al. (36).

We have recently constructed a subcritical water extractor by modifying a commercially available unit (37). With subtle changes, this unit can also be used for $SC-CO_2$ extractions. Our philosophy toward using subcritical water is that it is a complimentary fluid to $SC-CO_2$ with respect to environmental compatibility. Providing analyte stability is not a problem, subcritical H_2O or $SC-CO_2$ have the potential to extract most analytes. Recently we have demonstrated this principle by extracting nonpolar, organochlorine pesticides from fruit matrixes.

Enhancing Selectivity in SFE

Initial expectations of some analysts for analytical SFE were that it would be a "magic" bullet with respect to sample preparation prior to analysis. Therefore, their expectations were that SFE would produce an extract relatively free of coextractives that could be directly analyzed for a target analyte. This was found to be true in some cases, but these have in time, proven to be the exception rather than the rule. The above expectations ignore the fact that there are also some similarities between SFE and selective extraction with liquid-based solvents. However, SFE can be made potentially more selective than liquid extraction, because the density or solubility parameter (38) can be varied with the extraction pressure or temperature. However, when this principle is applied for sample cleanup, problems arise, particularly when extracting food matrixes that have appreciable quantities of lipids. Here the solubility trends and threshold pressures are very similar for many compounds (38) as a function of temperature and pressure, limiting the fractionation of the unwanted components from the target analytes.

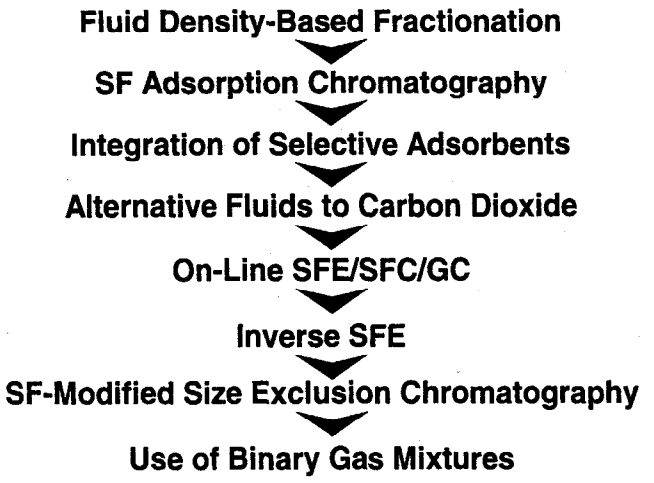


Figure 4. Development sequence for supercritical *fluid-based sample cleanup methods.*

This problem has been a major focus of our research and has let us on the odyssey depicted in Figure 4 to find the "ideal" sample cleanup method that could preferably be integrated with SFE. Space precludes a thorough discussion of all of these methods (in Figure 4), but all have found a niche in our method development research and have been practiced by others. Initial studies in our research group showed that a selective sorbent could be utilized, either in-line or in the SFE cell for selective retardation of lipid matter relative to pesticide analytes (39). The principle involved is a "supercritical" version of the low resolution, normal-phase chromatographic-based sample cleanup method cited in many pesticide residue protocols.

As noted by Randall (40), SC–CO_2 is a weak elutropic solvent, and one must be careful in selecting a sorbent, or tailoring its surface activity, so as to permit elution of the desired moieties. Consequently, sorbents used for fractionation in analytical SFE tend to be those associated with normal-phase liquid chromatographic applications, or those that have low energetic surfaces where permanent chemisorption of the analytes is absent. The following tabulation is a fairly comprehensive list of the materials utilized to date: alumina, silica gel, silicas, Florisil, celite, Hydromatrix, desiccant materials, modified silicas, adsorbent disks, and synthetic resins/foams. Our role in popularizing Hydromatrix as a universal sample preparation aid resulted in a patent (41), but use of the sorbent has been plagiarized by several commercial companies.

Sorbent-based chemistry can be used in several modes when coupled with SFE. Figure 5 illustrates use of Hydromatrix as a sample dispersant, mild desiccant, and void volume filler in the extraction cell. Figure 5 also illustrates how alumina as a sorbent can be used in its traditional format after SFE to segregate the target analytes from fat coextractives that often plague the food analyst, or conversely, within the SFE cell to retain more polar target analytes that can then be eluted off the alumina. In the latter case, the isolated analytes can be removed from the alumina by increasing the extraction pressure/temperature, or by incorporating an organic cosolvent with SC–CO_2, or by simply emptying the cell and using a liquid to elute the target analytes off the alumina. Such methods have been used extensively by Maxwell and coworkers (42, 43) for the analysis of antibiotics in biological tissues. The above-

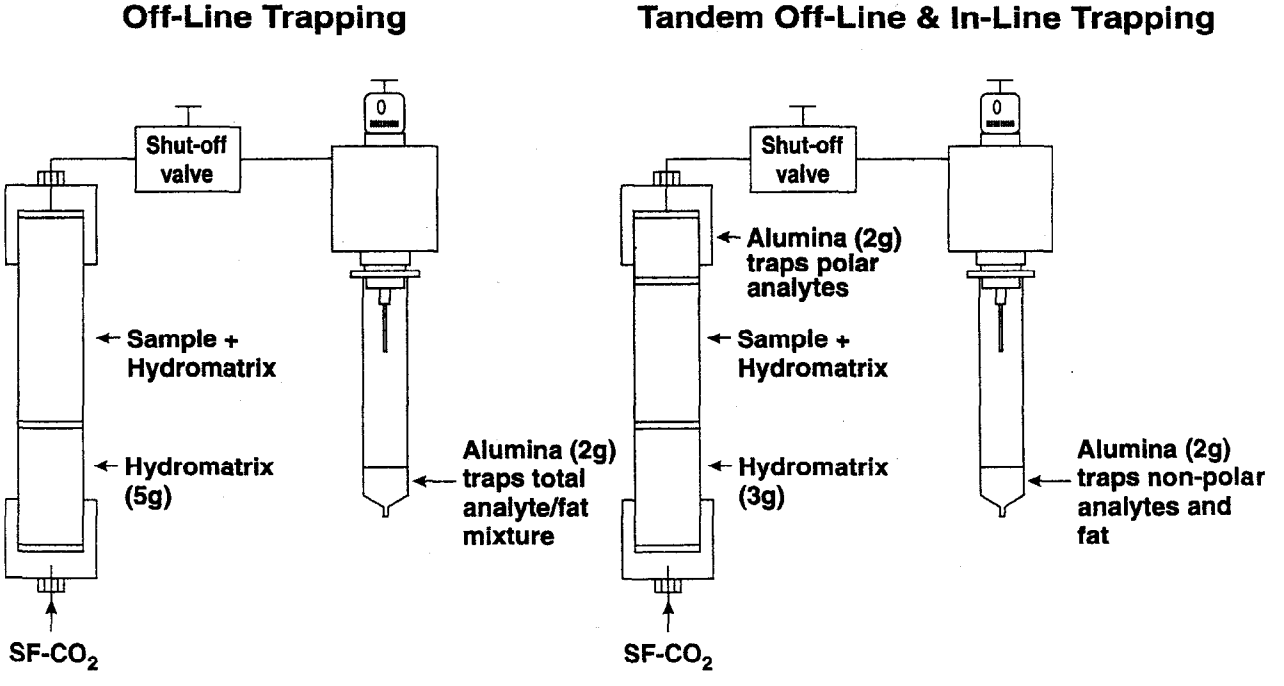


Figure 5. Supercritical fluid extraction configured for off-line and in-line analyte trapping.

cited use of alumina in the extraction cell to retain the desired analytes is an example of "inverse" SFE previously mentioned. Other examples of inverse SFE practiced by the author and others are separation of lipid matter from leucogentian violet in poultry tissue, reduction of interfering lipids from extracts containing cholesterol, cleanup of an extract containing aflatoxin M_1, and isolation of polymyxin B sulfate from neosporin cream.

Recently, we have developed a new selective supercritical fluid-based isolation method for pesticides in fatty food matrixes (44). This approach was derived from our studies on the influence of helium headspace on the solubilities of lipid moieties in SC–CO_2. Entrained helium in CO_2 can substantially reduce a solute's (analyte's) solubility in SC–CO_2 due to a decrease in the overall solubility parameter of the fluid and disruption of the CO_2 solvation shell around the solute. This trend has also been demonstrated for the effect of nitrogen on the solubility of caffeine in SC–CO_2 (45). This phenomena indicated to us that by using a fluid whose solubility parameter was less than CO_2's over the same range of pressure and temperature as SC–CO_2 (Figure 6), that selective extraction of target analytes, relatively free of lipids, might be realized. This is due to the fact that trace levels of target analytes (solutes) do not require a high solubility in SC–CO_2 to be extracted; therefore, they can be extracted free of large amounts of lipids if the proper extraction pressure and temperature, as well as the addition of the appropriate mole fraction of the second fluid, are utilized. Thus, it was found that 30 mole percent nitrogen in SC–CO_2 at 8000 psi and 60° or 80°C, yielded extracts with acceptably low lipid content and high organo-chlorine and phosphorus pesticide recoveries, along with gas chromatograms that were virtually identical to those obtained via conventional alumina column cleanup (44). We believe that the key to this selective extraction lies in the fact that the inclusion of N_2 along with CO_2 moderates the solubility of lipid moieties in the binary extraction fluid.

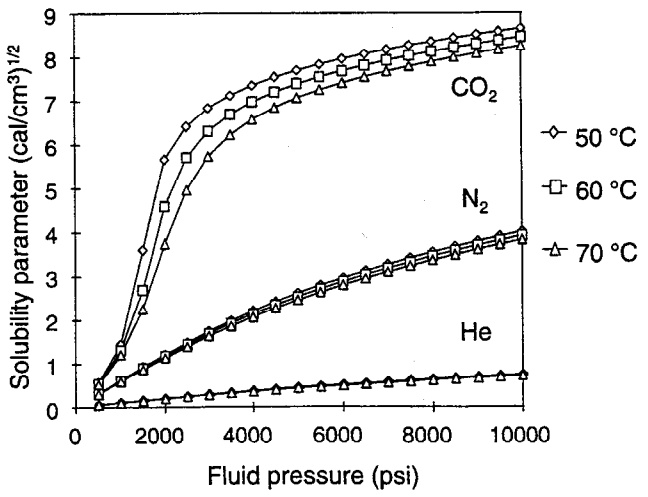


Figure 6. Solubility parameters for helium, nitrogen, and carbon dioxide versus fluid pressure.

Getting the Fat Out

One of the successful and commercially important areas for analytical SFE is determination of fat and oil levels in food and agricultural products. Traditional methods for fat analysis have incorporated a wide variety of solvents and pre-extraction preparation methods for different types of food matrixes. As shown in Table 2, the AOAC *Official Methods of Analysis* (46) lists 28 different solvent extraction methods that certainly promulgate use of organic solvents as well as confusion as to what is the accurate fat content in a food matrix. Such determinations have to a large extent been conducted on Soxhlet-type apparatuses previously referred to in this manuscript.

Experimental process studies conducted in our laboratories in the early 1980's allowed us to optimize SFE conditions for removing vegetable oils from seed matrixes (47, 48). This and additional processing studies to remove fat and cholesterol from meat matrixes (49) increased our knowledge as to how to prepare a high moisture-containing matrix for successful fat removal via SFE. Having such information and technique in hand allowed us to extend the concept for analytical determination of fat and oil levels in a variety of food and agricultural matrixes (50). This is a good example of the synergism we have enjoyed in our research effort between the process side of our studies and the analytical method development effort at the National Center for Agricultural Utilization Research.

However, simple gravimetric-based analytical SFE assay for fats in foodstuffs (51) were to be eventually questioned by us with the introduction of the new definitions and analytical protocols mandated by the Nutritional Labeling and Education Act (NLEA) in 1990 (52). These new analytical protocols for fat determination create considerably more effort and complexity for the fat analyst because they involve pre-extraction hydrolysis and an elaborate gas chromatographic (GC) analysis of the methyl esters of the constituent fatty acids comprising the fat moieties in the foodstuff. These steps, in addition to extraction of the sample,

Table 2. Fat analysis—AOAC methods[a]

960.39	933.05	945.48G	945.44
976.21	905.02	932.06	963.15
985.15	989.05	948.15	925.07
920.39B	938.06	986.25	920.177
920.39C	920.111A	925.32	920.172
945.18A	920.111B	948.22	963.22
945.38F	952.06	950.54	979.19

[a] *The Referee*, Vol. 17, No. 2, pp. 6–7, 1993.

have presented a severe challenge for integration into an SFE-based protocol.

To establish a baseline, we developed a method whereby all steps that were inclusive in the NLEA solvent extraction-based protocol were utilized in a procedure incorporating extraction with SC–CO_2, rather then the specified liquid solvent (53). This off-line SFE method utilized a sorbent disk to entrap the resultant lipid precipitate from a meat sample after acid hydrolysis of the meat sample, via filtration. The disk containing the precipitate was subsequently placed inside an extraction cell and the fat removed by SFE using CO_2. Trials on 2 different commercial SFE units indicated that the technique was not instrument dependent. Further, comparison of the results from the SFE procedure with those obtained via the traditional solvent-based protocol were equivalent for 9 different meat matrixes representing different levels of fat and types of meat. However, this procedure was exacting and difficult to reproduce in the hands of an unskilled analyst.

However, a development in our process studies, namely the enzymatic-catalyzed production of methyl esters of vegetable oils by coupling SFE with a *trans*-esterification reaction conducted in the presence SC–CO_2, proved applicable to the above analytical problem (54). For example, oil samples could be readily dissolved in SC–CO_2/methanol mixtures at pressures of 2500 psi and a temperature of 50°C to produce quantitative yields of fatty acid methyl esters (FAMES). This transformation was facilitated by passing the SC–CO_2/methanol mixture containing the dissolved oil over a supported lipase (Novozym 435) derived from *Candida antarctica*, which was placed downstream from the sample in the extraction cell. An example of the optimization of this enzyme-catalyzed reaction as determined by capillary supercritical fluid chromatography (SFC) is shown in Figure 7. Obviously, a pressure of 2500 psi and temperature of 50°C provides a complete conversion of the glycerides to the desired FAMEs, while maintaining enzymatic activity in the presence of SC–CO_2.

The excellent and reproducible yields of the FAMEs, when compared to results achieved using classical FAME derivatization methods, indicated that we had another method for conducting NLEA-based fat analysis. When this off-line SFE method was applied for speciated fat analysis in meats, good agreement was obtained with those values derived from solvent-based NLEA methods (55). This base method was further refined and expanded by incorporating it into an automated sequential SFE/GC system (55) in which the extraction/reaction were achieved on the automated SFE system, and by using a robotic arm interface, transferring the derivatized extracts onto the sampling tray of the GC for automated, overnight, unattended analysis. Results obtained on the above-mentioned meat samples using this system were also consistent with the values obtained by NLEA analysis. Additional studies by Snyder et al. (56) on model lipid compounds, such as phospholipids and cholesteryl esters, showed that the enzyme-catalyzed reaction worked equally well in determining the fatty acid contribution from these species. A flow diagram charting the development and

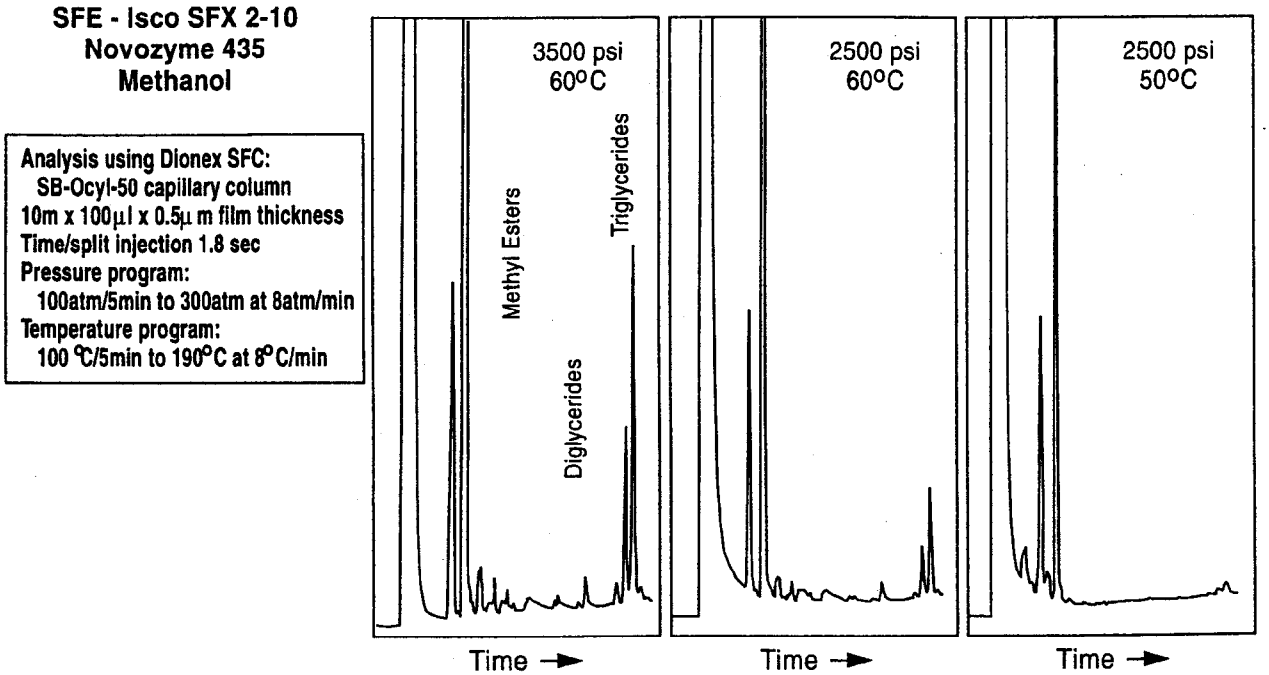


Figure 7. Supercritical fluid chromatograms showing the conversion of vegetable oil to monoglyceride as a function of reaction pressure and temperature in SC–CO_2.

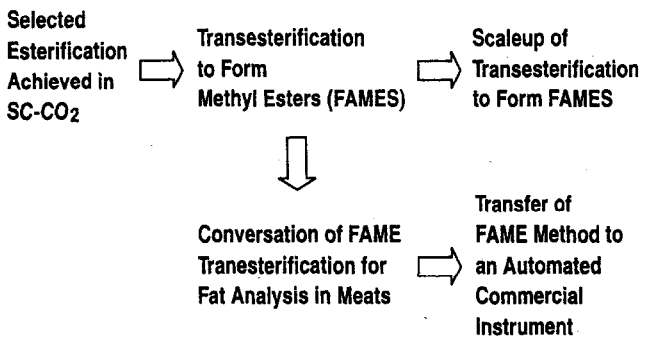


Figure 8. Development and utilization of lipase reaction in SC–CO_2 at NCAUR.

application of this enzymatic reaction in SC–CO_2 is shown in Figure 8.

Further exploitation of this combination of extraction/reaction chemistry in SC–CO_2 is shown in Figure 9. As indicated, it has been recently applied to oilseeds and cereal products and to characterize the fatty acid content of soapstock, a side stream product from the refining of vegetable oils that has commercial utilization (57). Comparison with the official American Oil Chemists Society (AOCS) method for fatty acid content in soapstock (58) have been excellent and the method has reduced the total analysis time by 50%. Furthermore, solvent use in the AOCS method was 575 mL; the supercritical fluid extraction/reaction scheme using enzymatic catalysis requires only 1.8 mL solvent! Recently, the above-described automated method has been applied to screen for the activity of lipases in conducting FAME *trans*-esterifications in the presence of SC–CO_2, thereby "inversing" the typical analytical use of the procedure (59).

Summary

In conclusion, I hope I have shown how we have contributed to the growth and acceptance of SFE as an analytical technique. Developments in our laboratory have closely paralleled studies by my colleagues in their research groups, and I want to acknowledge their assistance and encouragement over the years. I believe our approach at the NCAUR has benefitted considerably from our findings in process development and I encourage analysts to consult the engineering literature in this field whenever possible. Process as well as analytical developments in the field of supercritical fluid technology will be the focus of the 8th International Symposium on Supercritical Fluid Chromatography and Extraction on July 12–16, 1998, in St. Louis, Missouri.

Finally, I want to pay particular acknowledgment to my colleagues at NCAUR without whom many of my conceptual ideas would not have been brought to reality. Thanks go to Janet Snyder, Scott Taylor, Fred Eller, James Johnson, Gary List, and Jeffrey Teel. Their efforts aided by the assistance of many capable postdoctoral fellows and visiting scientists, including Zhouyao Zhang, Ki-Souk Nam, John France, Russell Holliday, Michael Jackson, Eila Jarvenpaa, and Fabio Favati have made this research and development of analytical SFE possible.

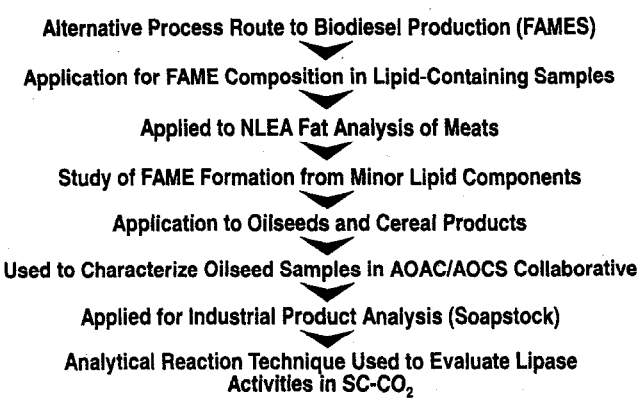


Figure 9. Utilization of lipase-initiated *trans*esterification in analytical and process SFE.

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Reprinted from the Journal of AOAC International
Volume 81, No. 1, 1998